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Fusion PCR, a One-Step Variant of the “Megaprimer” Method of Mutagenesis

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Two methods of producing fusions of two DNA stretches are currently in

use. The classic method involves the use of restriction enzymes and ligase. Recently, several methods have been published that describe how two DNA molecules were fused using polymerase chain reaction (PCR). In some, such as the PCR third irrelevant enzyme site (TIES) method (8), restriction enzymes and ligase are still needed. However, in most of these new

methods, no further processing of the formed DNA after PCR is needed. The latter methods include splicing by overlap extension (4) and the megaprimer method (7). A variation of the megaprimer method, using primers with different melting temperatures, was described that allows the fusion product to be generated in one tube after two rounds of PCR (5). I report an improved PCR protocol that condenses two fragments in only one step. In principle, it is a three-primer PCR in which the concentration of one of the primers is suboptimal. Variations of this protocol were used to generate plasmids without additive cloning and to generate precise deletions.

The method is outlined in Figure 1. All three primers indicated are added to the reaction mixture. Initially, however, the PCR using primers 1 and 2 will be prominent because of the smaller size of the product. This will generate a molecule with the complementary sequence of primer 2 at the 3' end. Because this sequence can hybridize with the other DNA molecule, a few hybrid DNAs will be formed that comprise both original sequences. The concentration of primer 2 will decrease rapidly and will no longer be sufficient to drive the PCR. In subsequent rounds of PCR, primers 1 and 3 are used to amplify the fusion generated in the earlier steps.

Because most polymerases add a single nucleotide, preferably an A (1), on the 3' end of the newly synthesized strand, it is wise to choose primer 2 in such a way that an extra A 3' to the complementary sequence of primer 2 will correspond to an A in the sequence of the second DNA fragment. Because no polymerase adds As to all strands, the fusion PCR method will even work with primers not specially designed to end before an A.

In Figure 2A, two fusions are shown, both of which are fusions of the *codA* gene of *E. coli* (2). In the left part of the figure, *codA* is fused 5' of the *A. terreus* BSD gene (6). Various concentrations of primer 2 relative to primers 1 and 3 are used in a standard PCR. The PCR protocol was developed for use with the PCR Core Kit (Catalog No. 1578553; Boehringer Mannheim GmbH, Mannheim, Germany), a kit based on *Taq* DNA polymerase. The

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final volume was 60 μ L, the total amount of oligonucleotides 1 and 3 was 30 ng each, dNTP concentration was 50 μ M, and a total of 15 ng of each plasmid was added. Thirty-five cycles were performed on an Omnigene[®] Cycler (Hybaid, Middlesex, England, UK), with steps of 1 min at 95°C, 1 min at 59°C and 2.5 min at 72°C. The molecular ratios between the external primers and primer 2 were 1:1 in lane 1, 10:1 in lane 2, 100:1 in lane 3, 1000:1 in lane 4 and finally 10 000:1 in lane 5.

In lane 1, in which all three primers were added in equal amounts, a major band of 1320 bp is seen corresponding to the PCR product of primers 1 and 2. A minor band of 1703 bp can also be seen corresponding with the predicted length of the *codA*-*BSD* fusion gene. In lane 2 with only 1/10 the amount of primer 2, the balance of the bands produced has shifted markedly towards the larger band. Other ratios do not give rise to full-length product, as can be seen in the following lanes, perhaps because of too few molecules produced with the hybridizing 3' end.

On the right-hand site of the figure (lanes 6–10), a fusion of the hygromycin-resistance gene (3) and *codA* is shown; because the differences in lengths are more pronounced than in the previous case (1011 and 2281 bp), the effects of the concentration of primer 2 are even more obvious. The same ratios of primers 1 and 3 vs. primer 2 were used. In this case, the smaller product is made almost exclusively when all three primers are used (lane 6). Again, the use of only one tenth of primer 2 gives the best results; here, almost all product is in the form of fusion sequences (lane 7).

Both fusion products were cloned, and the inserts of several of the resulting clones were sequenced, all of which had the correct sequence.

This protocol can also be used to generate deletions, a special case of fusion in which both fragments are originally linked by intervening DNA. The application of deletion mutagenesis is shown in Figure 2B. In this experiment, the gene for vascular endothelial growth factor (VEGF) isoform 189 was changed into isoform 121 by deletion of 204 bp corresponding to the naturally occurring splice variants. In the control

lanes, the PCR products of VEGF₁₂₁ and VEGF₁₈₉ are shown (lanes 1 and 2, respectively). To prevent contamination of subsequent PCR experiments, a mutant VEGF₁₂₁ distinguishable from the wild-type was used. In the third lane of Figure 2B, the result of a deletion PCR on VEGF₁₈₉ is shown. The length indicates that a deletion has indeed taken place. The product was cloned, and 8 clones were sequenced. All of these were derived from VEGF₁₈₉ because the sequence was wild-type. All 8 had correctly linked the DNA, and no mis-

takes were found.

Another application is the construction of plasmids consisting of a PCR fragment directly linked to a vector. This is but a variant of normal fusion PCR, in which one fragment contains an origin of replication and a selectable marker, but it will eliminate almost all cloning. The only step still necessary after PCR is the circularization of the newly formed linear molecule and the subsequent transformation into *E. coli*. This kind of fusion PCR works also with polymerases that add an extra A

Figure 1. Principles of fusion PCR. The diagram illustrates one small and one large molecule. All oligonucleotides and oligonucleotide-derived DNAs are drawn in gray; the original DNA is in black. The three oligonucleotides used are: [1] the primer that will be incorporated at the 5' end in the final DNA molecule. Extra restriction sites can be added for easy cloning; [2] the primer that will be used to fuse the two DNAs. Its sequence is composed of both the 3' end of the left DNA molecule and the 5' end of the right DNA molecule. On the border of these two moieties, extra restriction sites can be incorporated without apparent loss of efficiency; and [3] the primer that will direct the sequence of the 3' end of the final molecule. Again, extra restriction sites can be added to the primer. The protocol can be visualized in three steps: (i) the faster reaction using primers 1 and 2 will dominate at first. The PCR will incorporate the 5' end of primer 2 (antisense); (ii) the newly synthesized sequence can bind to the second DNA molecule and prime the fusion; and (iii) as the concentration of primer 2 drops, the reaction using primers 1 and 3 will produce more of the fusion.

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Figure 2. Examples of fusion and deletion. (A) Final amount of fusion product depends on the ratio of primers 1 and 3 vs. primer 2. Lanes 1–5: PCR on two plasmids coding for the codA and BSD genes. Lanes 6–10: PCR on plasmids coding for hygromycin resistance and codA. “B”: band corresponding to the codA-BSD fusion product; “H”: hygromycin-resistance gene; “HC”: fusion of the hygromycin-resistance gene and codA; lanes M: marker. (B) Deletion PCR on VEGF₁₈₉. Lane 1: PCR on cDNA for mutant VEGF₁₂₁. Lane 2: PCR on cDNA for VEGF₁₈₉ with two primers (1 and 3). Lane 3: PCR on cDNA for VEGF₁₈₉ with all three primers.

nucleotide. Even if 75% of its product were extended, 6.25% of the product would still be blunt-ended on both sides and be ligatable into closed circular DNA.

The method described here is a fast, dependable method to fuse or delete genes in the shortest possible time. In addition, it is very accurate up to the single-nucleotide level, independent of the polymerase used. Because of its lack of restriction steps and the use of only three oligonucleotides, it is also very economical.

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